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THE REDUCTION OF ANTIGENICITY OF HETEROLOGOUS ANTILYMPHOCYTE SERUM WITH ACID TAKA-PROTEASE

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SUMMARY

Horse anti-dog and anti-mouse ALS were digested for varying intervals with acid Taka-protease, a proteolytic enzyme. A progressive reduction in antigenicity was demonstrated, as determined by *in vitro* precipitin reactions with standard antisera and by passive cutaneous and systemic anaphylaxis tests. Leukoagglutinins and lymphocytotoxins were conserved in anti-dog ALS but there was a marked diminution in anti-platelet and anti-erthrocyte activity. Both anti-red cell and anti-white cell antibodies of anti-mouse ALS were reduced. Digestion appeared to completely degrade some protein fractions such as albumin and to alter the immunoglobulin molecule, possibly by cleavage of the terminal portion of the Fc fragment. Digestion of ALS with Taka-protease may prove to be a useful procedure particularly since it was demonstrated that immunosuppressive potency was partially retained. For further evaluation a better standardized Taka-protease will be required.

INTRODUCTION

Digestion of proteins in order to reduce their immunogenicity is a well-known commercial procedure. Since heterologous anti-lymphocyte serum (ALS) and its globulin derivatives (ALG) have human use, it was natural for this approach to be tried in an effort to decrease the risk of side effects consequent to foreign protein sensitization (Kashiwagi *et al.*, 1968; Amemiya *et al.*, 1970). Anderson *et al.* (1965) described peptic digestion with the preparation of $F(ab')_2$ fragments and further cleavage to Fab′ with cysteine hydrochloride. Both digestion products lost much of their lymphocytotoxic activity and to a lesser extent the leukoagglutinating action; furthermore, they no longer had a potent immunosuppressive effect when given to intact animals. Probably as a result of these discouraging findings, the matter apparently has not been explored further.

In this investigation, horse anti-lymphocyte serum was digested with an enzyme called

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Taka-protease that is derived from the mould *Aspergillus oryzae* (Crewther & Lennox, 1950) and which may have a different site of splitting action than pepsin or papain. With this digestant, the antigenicity of the ALS was reduced or eliminated. At the same time, the titres of leukoagglutinins and lymphocytotoxins remained relatively high while the undesirable thromboagglutinins and haemagglutinins were disproportionately reduced. Some immunosuppressive effect was retained.

METHODS

Preparation and standardization of Taka-protease

Preparation of acid Taka-protease. A relatively pure solution of acid Taka-protease was prepared from commercial Taka-diastase (Parke-Davis & Co. Detroit, Michigan) by auto-digestion, wherein the protease present in the crude mixture inactivates the other undesired enzymes at an acid pH (Amano, Isojima & Fujio, 1953, 1954). 50 g of commercial Taka-diastase were dissolved in 500 ml of distilled water and stirred vigorously at room temperature for 2 hr and for an additional 12 hr at 4°C. After acidification with 1 N HCl to pH 3·3, the solution was incubated at 37°C for 1 hr. Auto-digestion was terminated by adjusting the pH to neutral. The resulting 'acid Taka-protease' was stored at 4°C.

Quantitation of proteolytic activity. The enzymatic activity of Taka-protease was quantitated by reacting it with a standard substrate of normal horse serum (Amano *et al.*, 1953), measuring the liberation of the amino acids tyrosine and trytophan with the method of Folin & Ciocalteu (1927). The substrate was prepared by mixing 2·0 ml of normal horse serum previously dialysed against distilled water for 24 hr at 4°C and diluted to a concentration of 1·0 g% with an equal volume of 0·1 N, pH 3·2, acetate buffer. An 0·5-ml volume of Taka-protease was added and the solution incubated for 20 min at 38°C. Undigested protein was precipitated with 5 ml of 10% trichloroacetic acid and the amino acid-containing supernatant was filtered. 0·5 ml of the filtrate was combined with 5 ml of alkaline copper solution and 0·5 ml of a 1 : 3 dilution of Folin-Ciocalteu reagent (Fisher Scientific Company, Fair Lawn, New Jersey). After heating in a 45°C water bath for 15 min, the solution was allowed to cool to room temperature before measuring extinction, expressed as optical density, with a Coleman spectrophotometer set to a wavelength of 660 mμ. Because the Taka-protease solution itself contained amino acid contaminants released by its auto-digestion, a control sample was prepared in which proteolysis of the substrate was prevented by reacting it with trichloroacetic acid prior to the addition of Taka-protease. Proteolytic activity was expressed by the formula:

$$\text{Activity (units/ml)} = 20 \times (\text{Test extinction} - \text{Control extinction}) \times \text{enzyme dilution.}$$

Digestion of ALS with Taka-protease

Horse anti-dog ALS. A single batch of acid Taka-protease with digestion activity of 6·0 units/ml was used for all the studies to be reported. Anti-lymphocyte serum was obtained from a horse which had received fourteen immunizations with canine spleen cell suspensions. The antibody activities of the crude ALS are given in Fig. 3. These titres were determined after diluting the ALS volume for volume with normal saline since this same dilution with Taka-protease and buffer was required for the actual digestion process to be described below. By making the adjustment, it became possible to accurately judge the effect of the digestion process upon the titres.

To each 180-ml batch of ALS submitted to digestion, one-tenth volume of 5% phenol was added as a preservative. The pH was then adjusted to 3·0 with 1 N HCl as recommended by Amano *et al.* (1954) and maintained at that acidity with 36 ml of 1 N acetate buffer at pH 3·0 as 100 ml of Taka-protease was added. While at room temperature (20°C), the mixture was diluted to 360 ml with distilled water and divided into nine aliquots. Approximately 30 min after the addition of Taka-protease, the enzyme-ALS solutions were warmed to 37°C and each sample incubated for a different time interval, ranging from 0 to 240 hr (Table 1). At the end of the various intervals, digestion was stopped by neutralization with 4 N NaOH. In those samples incubated for 72 hr or longer, there appeared a visible brown flocculant which was presumably denatured protein; this was removed was by centrifugation at 10,000 rpm for 30 min prior to storage of the specimens at −70°C.

Horse anti-mouse ALS. Commercial ALS (Microbiological Associates, Inc., Bethesda, Maryland) raised against Swiss Webster mice was digested for 72 hr with Taka-protease under the same conditions as described in the preceding section. The globulin fraction remaining after digestion was precipitated with 60% saturated ammonium sulphate and dialysed against tap water.

Characterization of digested ALS

Antibody activity. In the anti-dog and anti-mouse ALS the titres of agglutinating antibodies against canine or mouse erythrocytes, leucocytes, and thrombocytes, were determined with a microplate method (Cook Engineering Co., Alexandria, Virginia). To serial two-fold dilutions of the crude ALS or its digested products were added equal volumes (0·025 ml) of the appropriate cell suspension. In all anti-dog ALS studies, cells were obtained from the heparinized blood of a dog kept as a chronic blood donor through the period of the investigation. Mouse cells were from Swiss Webster strain animals.

The haemagglutinin and leukoagglutinin determinations were carried out according to standard techniques, reading the end points microscopically. The white cells for the latter test were obtained by the dextran sedimentation method (van Rood & van Leeuwen, 1963). A suspension containing 40,000–50,000 platelets/mm^3 was used for the thrombo-agglutinin titrations. After 6 hr incubation at room temperature the plates were read with the naked eye. Lymphocytotoxins were assayed by a modification of the microdroplet method of Terasaki & McClelland (1964), using lymphocytes purified from buffy coat with glass bead adherence and distilled water haemolysis. Complement was derived from the blood of the white cell donor.

Protein content. The protein concentration of the ALS and the products of its digestion were measured with a Hitachi-Perkins spectrophotometer at a wavelength of 750 mμ after reacting the substrate with Folin-Ciocalteu phenol reagent (Kabat & Mayer, 1964).

Electrophoresis and immunoelectrophoresis. Electrophoretic separation of protein components was carried out with the Beckman Microzone cell unit. Electrophoresis was for 24 min at 225 V.

Scheidegger's (1965) micromethod was employed for immunoelectrophoretic analysis. A potential gradient of 5 V/cm was applied for 120 min. The antisera selected were those used for the Ouchterlony immunodiffusion tests of antigenicity (see below).

Antigenicity. In vitro examination of antigenicity was with the Ouchterlony immunodiffusion test, as modified by Torisu *et al.* (1970). This technique measures the reactivity of ALS or its digestion products against standard antisera raised against horse serum or its

TABLE 1. Lymphocytotoxin and protein contents of crude ALS and the products of its digestion with Taka-protease

	Crude ALS	Duration of digestion (hr)								
		0	6	12	24	48	72*	96*	120*	240*
Lymphocytotoxin titre	1 : 512	1 : 512	1 : 128	1 : 128	1 : 128	1 : 128	1 : 128	1 : 128	1 : 128	0
Volume recovered (ml)	40·0	40·5	45·0	42·0	42·0	42·5	28·0	33·5	33·0	33·0
Total activity† (units)	20500	21000	5760	5370	5370	5430	3580	4290	4240	0
Total protein‡ (mg)	3650	4170	2510	2510	2540	2220	932	887	918	917

* All values recorded after 72 hr or more of digestion were measured in the supernatant obtained by centrifugation, hence the sudden fall in protein content as well as volume.

† The total antibody activity is the product of the reciprocal of the lymphocytotoxin titre and the volume of supernatant recovered.

‡ These values represent a total to which several heterogenous proteins contribute. The reason is that the determinations of protein concentration, and thus the calculated total protein content, cannot differentiate between horse serum protein, its degradation products, and the protein-containing enzyme itself.

fractions. Serial two-fold dilutions of digested ALS were placed in the peripheral wells; the central well was filled with one of four antisera: guinea-pig antisera to horse IgG or to the T-equine fraction and rabbit antisera against normal horse serum or IgG. After 48 hr of diffusion at room temperature all precipitin bands were recorded. The antigenicity was expressed as the greatest dilution of ALS giving a visible reaction with that antiserum. Both of the two rabbit antisera (Hyland Laboratories, Los Angeles, California) were found

TABLE 2. Passive systemic anaphylaxis with crude and digested horse ALS using rabbit anti-horse serum antiserum

Materials for antigen injection	No. of animals	Protein dose (mg)	Results
Crude ALS	2	1·0	3+, 3+
ALS digested for 0 hr	2	1·0	3+, 2+
ALS digested for 12 hr	3	60·0	2+, 2+, 2+
ALS digested for 48 hr	3	60·0	1+, 1+, 1+
ALS digested for 96 hr	1	30·0	—

3+ = Death after typical anaphylactic syndrome; 2+ = convulsions, dyspnoea and coma but with recovery; 1+ = cough, irregular respirations, urinating, scratching, and rubbing.

TABLE 3. Passive cutaneous anaphylaxis with crude and digested ALS

		Area of dye spot (mm^2)			
			ALS digested for:		
Antiserum	Dilution	Crude ALS	0 hr	48 hr	96 hr
Rabbit antiserum to normal horse serum	1 : 50	2810	2810	0	0
	1 : 100	1670	1510	0	0
	1 : 500	700	960	0	0
	1 : 1000	570	660	0	0
	1 : 2000	420	310	0	0
	1 : 4000	190	130	0	0
Rabbit antiserum to horse IgG	1 : 250	910	1590	0	0
	1 : 500	30	1070	0	0
	1 : 1000	3	570	0	0
	1 : 2000	Trace	420	0	0
	1 : 4000	0	280	0	0
	1 : 8000	0	0	0	0

to have a titre of 1:6400 when tested against serial dilutions of normal horse serum containing 7 g% protein. Guinea-pigs were innoculated with IgG or T-equine globulin prepared from a single horse using the method of Oriol, Binaghi & Boussac-Aron (1968); the harvested immune sera reacted at a 1:800 dilution with the undigested ALS.

Two *in vitro* tests of anaphylaxis were conducted using the rabbit antisera described

TABLE 4. Anti-white cell and red cell titres, protein contents, and antigenicities of the ALS, digested ALS and normal horse serum used in the skin graft experiments shown in Fig. 5

	Titres			Antigenicity			
				Rabbit		Guinea-pig	
	Lymphocytotoxin	Leukoagglutinin	Haemagglutinin	Anti-horse serum	Anti-horse IgG	Anti-horse T-equine	Anti-horse IgG
Horse anti-mouse ALS	1 : 256	1 : 1024	1 : 2048	2560	640	320	640
Digested horse anti-mouse ALS	1 : 16	1 : 128	1 : 64	320	320	< 10	< 10
Normal horse serum	0	0	1 : 64	2560	640	320	640

	Protein content (g%)	Beta- and gamma-globulin/dose
Horse anti-mouse ALS	6·42	13·3 mg (0·25 ml)
Digested horse anti-mouse ALS	1·22	14·8 mg (0·50 ml)
Normal horse serum	6·17	17·4 mg (0·25 ml)

above to passively sensitize guinea-pigs to crude horse serum protein or purified horse IgG. In the *passive systemic anaphylaxis test* (Kabat & Landow, 1942) guinea-pigs weighing 250–300 g were injected intraperitoneally with 0·5 ml of antiserum 48 hr before the intravenous administration of 1 ml of either unaltered or digested ALS, the protein content of which was controlled by appropriate dilution. The reactions exhibited by the animals were graded from 0 to 3+ (Table 2).

The *passive cutaneous anaphylaxis test* of Ovary (1958) allowed more precise quantitation of reactions. Guinea-pigs weighing 300–350 g were innoculated intradermally with 0·1 ml of the same rabbit antisera, in six different dilutions (Table 3). Three hours later 1·7 mg of digested or undigested ALS in a 0·5-ml volume and 0·5 ml of 1·0% Evans' blue dye solution were injected intravenously. The area of the dye spots produced in the skin was measured after killing the animals.

Tests of immunosuppression. Swiss Webster mice received skin grafts from donors of the same outbred strain using the method of Billingham & Medawar (1951). On the day before grafting and on postoperative days 1, 3 and 5, three groups of five mice each were injected subcutaneously with either 0·25 ml of normal horse serum, 0·25 ml crude commercial ALS or 0·50 ml digested ALS. The double volume of digested ALS was to provide approximately the same dose of the combined beta and gamma fractions as in the normal horse serum and the unaltered ALS (Table 4).

RESULTS

Alterations in protein constituency

For the first 48 hr of digestion the mixture of anti-dog ALS and Taka-protease was a viscous and grossly homogenous fluid that resembled plasma. After this time, it 'clotted' with visible strands and with the consistency of gelatin. In all the samples after 48 hr centrifugation was carried out initially and only the supernatant retained for analysis of protein content and antibody titre. Consequently, there was a sharp reduction in both the volume recovered and the total protein of all the samples studied after 2 days. At these times only about a third of the protein originally submitted to digestion remained.

Although grossly visible changes in the mixture did not appear until after 48 hr, it was clear from the electrophoretic patterns (Fig. 1) that significant alterations in composition of the digested ALS had nevertheless occurred long before this time. Even when the digestion was terminated by neutralization immediately after the addition of enzyme, there was a modest decline in the albumin peak. Prior to incubation, but after 30 min at room temperature, there was further truncation of the albumin spike as well as diminution of the alpha peak. After 6 hr of incubation at 37°C (Fig. 1), the most prominent changes were in the albumin and gamma-globulin regions. From 12 hr onward (Fig. 1) only a single broad peak having an electrophoretic mobility in the range of beta- and gamma-globulin persisted (Fig. 1).

Because digestion may have altered the electrophoretic migrations of specific protein moieties these findings were re-examined with immunoelectrophoresis (Fig. 2). Using rabbit antisera directed against normal horse serum (Fig. 2, top left), the albumin disappeared first (at about 6 hr), followed by a decrease in the number of identifiable precipitin lines from 24 hr onward. At 96 and 240 hr the persistence of IgG was demonstrated with specific rabbit anti-IgG antisera (Fig. 2, top right). With the less sensitive but more specific

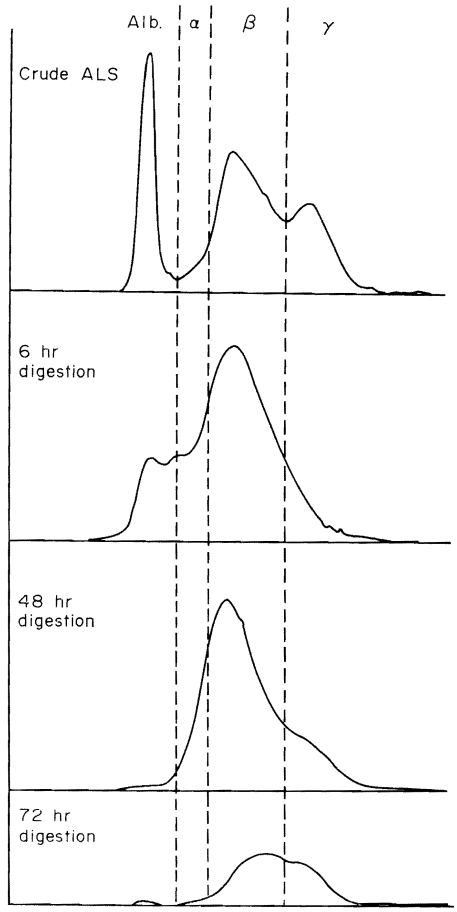

FIG. 1. Electrophoretic patterns of crude ALS and three representative products of its digestion with Taka-protease. The divisions of the curves into albumin, and α, β, and γ globulin regions are only approximations since the proteolytic process may have significantly altered the electrophoretic mobilities. The concentrations of protein and the volumes applied were variable; the curves, therefore, are not quantitative.

guinea-pig anti-T-equine and anti-IgG antisera, a T-equine globulin precipitin line (Fig. 2, bottom right) was faintly visible at 6 hr but absent thereafter. There was a similar disappearance of IgG within 6 hr (Fig. 2, bottom left), as measured by guinea-pig antiserum.

Antibody activities of digested ALS

Anti-dog ALS. Taka-protease digestion of ALS for less than 240 hr had little effect on the leukoagglutinin titres. These were not altered at all for the first 48 hr and fell only one tube in the several subsequent days (Fig. 3). There were moderate falls in the cytotoxin titres with a two tube reduction in the first 6 hr. However, this did not change further in the next 6 days. All the antibody determinations carried out after 48 hr were performed on the supernatant rather than on the total mixture. In spite of this, the anti-white cell

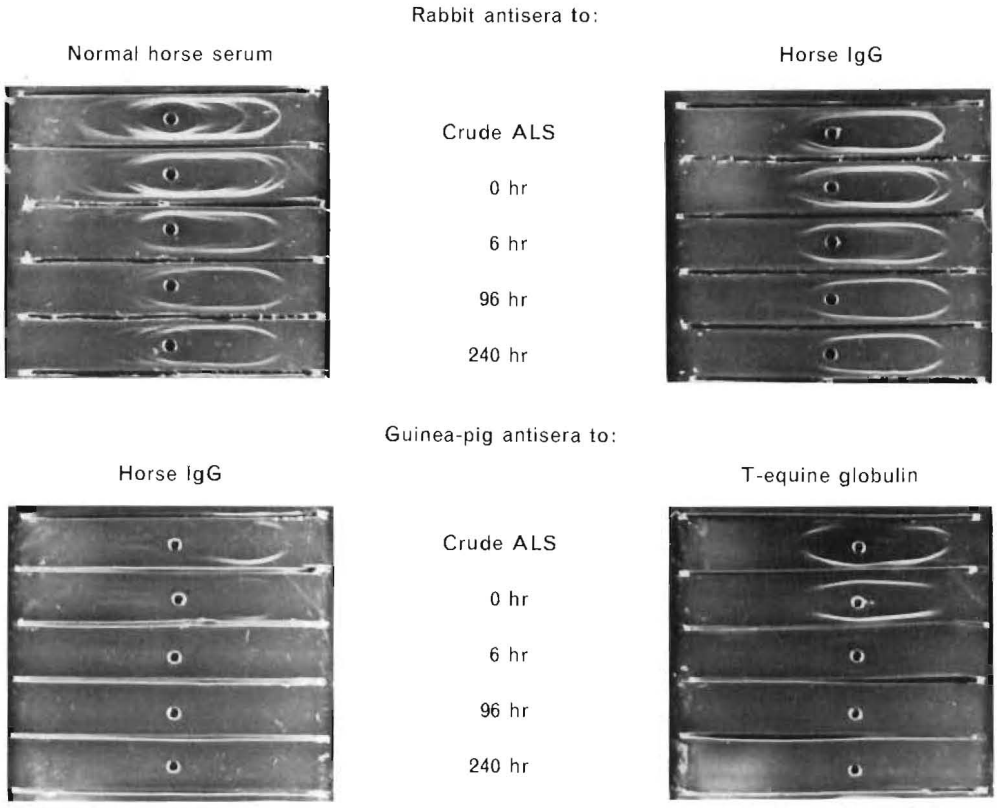

FIG. 2. Immunoelectrophoresis of crude and digested ALS products with antisera raised in rabbits (top panels) and guinea-pigs (bottom panels). The wells, reading from top to bottom on each plate, were filled with crude ALS or ALG digested with Taka-protease for 0, 6, 96, or 240 hr at 37°C. The troughs of each plate were charged with the antiserum designated above each panel. Note that the rabbit antisera produce well-defined precipitin bands when reacted with all ALS samples. However, the antisera obtained from guinea-pigs no longer could identify horse IgG after 6 hr digestion and T-equine globulin was scarcely identifiable at 6 hr and could not be detected in all subsequent samples. The changes evident in the '0 hr' specimens are the result of proteolysis occurring during the 30-min interval at room temperature prior to the initiation of incubation.

antibody titres were not reduced. The retention of titres despite a three- to four-fold reduction in total protein upgraded the relative antibody activity expressed on the basis of protein weight (Table 1).

To ensure that the lymphocytolytic activity measured was related solely to specific antibody and not augmented by non-specific lysins or toxic components released by proteolysis, samples of ALS digested for 0, 12, 48 and 120 hr were precipitated with 60% ammonium sulphate (Rowe, 1962), then dialysed against tap water and isotonic saline solution. When expressed as activity per mg of protein, virtually all of the lymphocytotoxins were recovered with the precipitated protein.

In contrast to the anti-leucocyte activities, the haemagglutinin titres declined precipitously during the first 6 hr and then gradually recovered, although never approaching

the pre-digestion level (Fig. 3). There was a similar effect on anti-platelet antibodies. The crude ALS possessed a thromboagglutinin titre of 1 : 64. When the serum was tested after acidification and addition of Taka-protease, but before incubation at 37°C, the titre had already fallen to 1 : 4 (Fig. 3), suggesting that anti-platelet antibodies had been altered or inactivated either by acidification or by digestion occurring during the 30-min interval at room temperature prior to incubation. That the latter effect contributed substantially

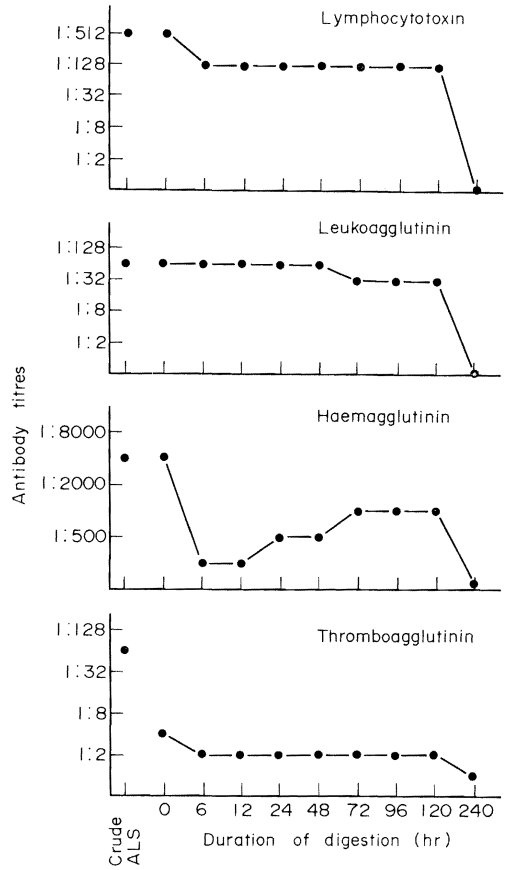

FIG. 3. Changes in antibody activities of horse ALS digested with Taka-protease. The crude ALS was diluted so that its concentration was identical to that present in the enzyme–substrate mixture before the initiation of digestion (see text). Note the preferential preservation of anti-white cell activity in contrast to the marked decline in haemagglutinins and thromboagglutinins.

more to the reduction in titre was shown by acidifying an aliquot of ALS, adding Taka-protease (in the same enzyme/ALS ratio as that used in the previously described digestion experiments), and allowing it to stand at room temperature for 1 hr before restoring the pH to neutrality. The thromboagglutinin titre was reduced by a factor of 32, whereas when distilled water was substituted for the Taka-protease, the anti-platelet activity was

reduced only four-fold. Further slight reductions in thromboagglutinin activity were noted during digestion at 37°C (Fig. 3).

Anti-mouse ALS. In contrast to the findings with anti-dog ALS, the titres of all measured antibodies fell precipitantly during the 72 hr of digestion, and without any obvious selective sparing of the lymphocytotoxins and leukoagglutinins (Table 4).

Changes in antigenicity

In vitro measures. After digestion of either horse anti-dog or anti-mouse ALS for 6 and 12 hr, respectively, the reactions of guinea-pig antisera directed against the horse T-equine

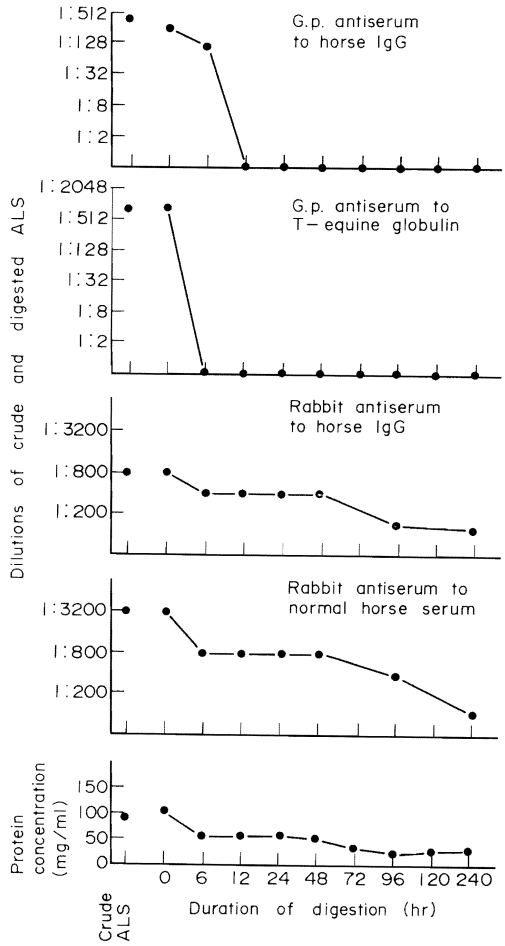

FIG. 4. Loss of antigenicity occurring during the course of Taka-protease digestion of ALS, as measured by immunodiffusion. The central well of the Ouchterlony plate was charged with one of the four antisera indicated. The peripheral wells were filled with either crude or digested ALS, diluted as shown on the ordinate of each graph. The antigenicity of a sample to a given antiserum was therefore the greatest dilution of the sample which still reacted to give a visible precipitin line. It should be noted that the protein concentrations (bottom curve) of the ALS specimens subjected to dilution were variable; the rabbit antisera curves after 48 hr of digestion appeared to parallel the protein content.

and IgG fractions had been completely abrogated (Fig. 4). When tested against the more polyvalent rabbit antisera, the digested ALS showed a more gradual decline in antigenicity, never becoming negative (Fig. 4).

In vivo systems. 1 mg of crude anti-dog ALS produced a lethal reaction in guinea-pigs submitted to the passive systemic anaphylaxis test (Table 2). Animals receiving 60 mg of ALS for 12 hr showed severe reactions but survived. Digestion for any longer time yielded a product that produced few or no symptoms even when given in a protein dosage 30–60 times that found to be the lethal one for crude ALS.

Anti-dog ALS digested with Taka-protease for 48 and 96 hr did not evoke antigenic reactions in the passive cutaneous anaphylaxis test, despite significant responses to the control samples (Table 3).

Immunosuppression

The survival of skin grafts in the Swiss Webster mice is shown in Fig. 5, under treatment with normal horse serum, crude anti-mouse ALS and the digested ALS. The digested ALS had a severe loss of potency but it was still demonstrably immunosuppressive.

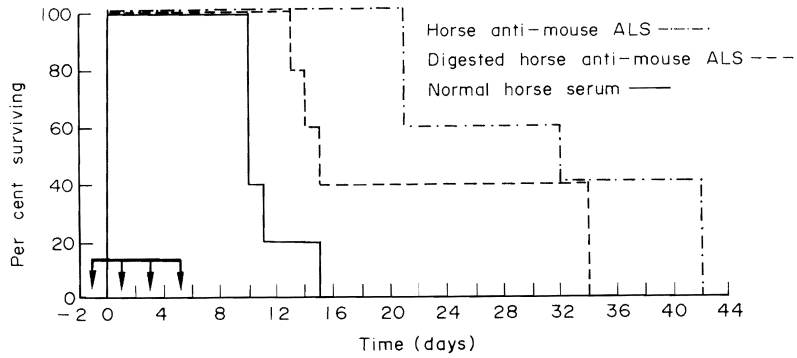

FIG. 5. Survival curves of skin grafts exchanged between outbred Swiss Webster mice. On the day before grafting and on postoperative days 1, 3, and 5 (arrows) each group of five mice received either 0·25 ml of normal horse serum, 0·25 ml of horse anti-mouse ALS, or 0·50 ml of the digested ALS, the titres, antigenicities and protein contents of which are given in Table 4. Note that the digested ALS retains some immunosuppressive potency.

DISCUSSION

With the clinical use of equine anti-toxins to diptheria and tetanus more than 70 years ago, the dangers of sensitization were soon appreciated, the antigenicity of heterologous proteins was recognized, and measures to circumvent the resulting hazards were explored.

Early efforts to alter or destroy the antigenic portion of hyperimmune sera by enzymatic digestion were invariably accompanied by severe losses of antibody activity. However, by carefully controlling the conditions under which pepsin was reacted with horse antiserum, Weil, Parfentjev and Bowman (1938) succeeded in preserving substantial antitoxin activity while reducing by a factor of 500 its ability to provoke anaphylactic reactions in guinea-pigs. They concluded that the digestion had altered the globulin molecule.

Shortly after, Coghill *et al.* (1940), acting on the premise that the antigenicity of pseudo-globulin resided in carbohydrate moieties, attempted despeciation—the removal of the

horse serum specificity—with several carbohydrate splitting enzymes. Only Taka-diastase was effective. Kass, Scherago & Weaver (1942) repeated these observations but concluded that a protease contaminant rather than a carbohydrase was the actual despeciating factor. Amano *et al.* (1953) confirmed that acid Taka-protease was the active component in the digestion of horse globulin by Taka-diastase. At about this time, research with Taka-diastase virtually ceased, partly because of the much greater convenience of carrying out studies with the pure enzymes pepsin and papain. The results of the latter digestion experiments have elucidated the structure of immunoglobulins as exemplified by Porter's (1959) molecular model of rabbit IgG with its heavy and light chain components (Fig. 6).

The enzymes pepsin and papain cause cleavage of the immunoglobulin molecule at locations which are sufficiently far apart so that the resulting fragments have different

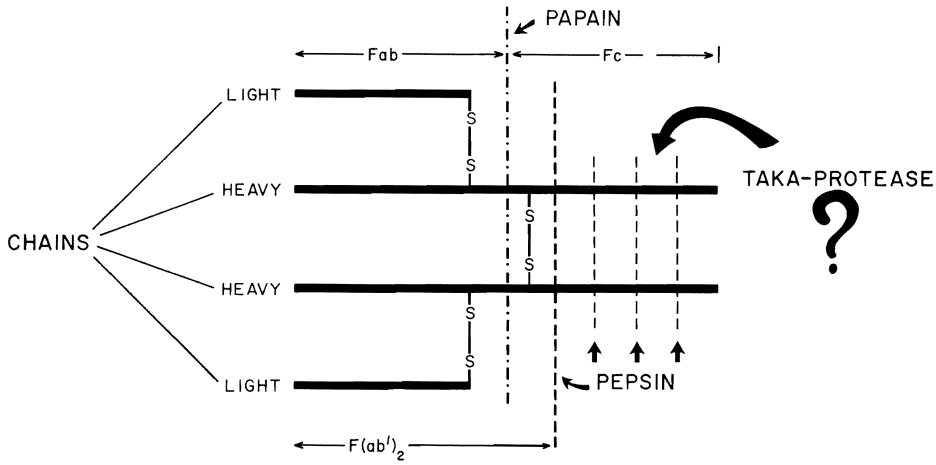

FIG. 6. Structure of rabbit IgG based on analyses performed by Porter (1959). Horse immunoglobulin is thought to have a similar configuration but with three disulphide bonds joining the heavy chains (Schultz *et al.*, 1965). The standard nomenclature for the fragments produced by peptic and papain digestion is given. Cleavage with papain yields two Fab units and the Fc portion. Pepsin splits the IgG molecule into an $F(ab')_2$ piece and several smaller fragments (dashed lines). The $F(ab')_2$ fragment may be split into two monovalent pieces by reduction as with mercaptoethanol. The site of action of Taka-protease is unknown, but is probably in the Fc region (see text).

physical, chemical and biologic characteristics. There was no *a priori* reason to assume that Taka-protease acted in exactly the same way as either of these digestants nor do the results in the present study support such an assumption. As would also have been expected with either pepsin or papain digestion, Taka-protease caused a significant reduction in antigenicity of ALS. This was demonstrable by immunodiffusion against antisera to normal horse serum or its IgG and T-equine fractions. Furthermore, guinea-pigs submitted to tests of passive cutaneous or systemic anaphylaxis, the experimental analogues of the clinical anaphylactic syndrome, either failed to respond or else exhibited less severe reactions to much larger quantities of protein than had been a lethal dose of crude ALS.

On the other hand, leukoagglutinin and lymphocytotoxin titres of anti-dog ALS were not very severely reduced by Taka-protease proteolysis. The total activities of these antibodies at first declined and then stabilized despite a considerable loss of protein following

more than 48 hr of digestion. With pepsin digestion, lymphocytotoxins in ALS were virtually eliminated (Anderson *et al.*, 1965).

An unexpected benefit of the Taka-protease process was the selective elimination of undesirable antibodies. Thromboagglutinins were inactivated by both acidification and digestion; a decline in haemagglutinin titre occurred only during incubation. In the absence of further information, it can only be postulated that anti-platelet and anti-red cell antibodies may differ significantly in structure or configuration from leukoagglutinins and lymphocytotoxins and, therefore, be more susceptible to denaturation or digestion.

The same selective degradation of thromboagglutinins and erythroagglutinins was not evident in digesting horse anti-mouse ALS. Here, there was a sharp and essentially parallel decline of anti-red cell and anti-white cell antibodies. Because of technical difficulties anti-platelet activity could not be measured.

The mechanism of action of Taka-protease on gamma-globulin is not known. Although our experiments were not designed to give this kind of information, some observations provided bits of circumstantial evidence. After elimination of the albumin by digestion for many hours, the residual broad peak in the IgG and T-equine regions could easily be identified as belonging to these fractions with specific rabbit antiserum. In contrast, guinea-pig antisera failed to identify these immunoglobulins as being either IgG or T-equine globulin. Oriol *et al.* (1968) have reported that the guinea-pig is capable of responding immunologically only to antigens at the terminal end of the heavy chain, but that rabbit antisera can detect determinants on both heavy as well as light chains. The persistence of rabbit antisera reactions at a time when the guinea-pig sera failed to cause precipitation would be consistent with cleavage at the terminal ends of the heavy chains as suggested schematically in Fig. 6.

The most practical question about ALS digested with Taka-protease was whether or not it retained its immunosuppressive qualities and to what extent. In the present study, the ability to prevent rejection of skin grafts was significantly reduced by Taka-protease, but it was not eliminated, as reported by Anderson *et al.* (1965) after pepsin digestion. In spite of this encouragement, formidable difficulties will have to be surmounted before Taka-protease can even be more thoroughly evaluated for its potential applicability to clinical medicine. Not the least of these is the need to have a standardized enzyme purified from the raw Taka-diastase to permit equivalent experiments to be conducted from batch to batch.

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REFERENCES

AMANO, T., ISOJIMA, S. & FUJIO, H. (1953) On the mechanism of despeciation of horse-serum globulin by Taka-diastase. *Med. J. Osaka Univ.* **4,** 255.

AMANO, T., ISOJIMA, S. & FUJIO, H. (1954) Mechanism of despeciation of horse-serum globulin by Taka-diastase. II. Further studies on acid Taka-protease. *Med. J. Osaka Univ.* **5,** 333.

AMEMIYA, H., KASHIWAGI, N., PUTNAM, C.W. & STARZL, T.E. (1970) Cross-reactivity studies of horse, goat, and rabbit antilymphocyte globulin. *Clin. exp. Immunol.* **6,** 279.

ANDERSON, N.F., CLARK, J.G., JAMES, K., REID, B.L. & WOODRUFF, M.F.A. (1965) Biological properties of antilymphocytic antibody and antibody fragments. *Advance in Transplantation* (Ed. by J. Dausset, J. Hamburger and G. Mathé), pp. 103–106. Munksgaard, Copenhagen.

BILLINGHAM, R.E. & MEDAWAR, P.B. (1951) The technique of free skin grafting in mammals. *J. exp. Biol.* **28,** 385.

COGHILL, R.D., FELL, N., CREIGHTON, M. & BROWN, G. (1940) The elimination of horse-serum specificity from antitoxins. *J. Immunol.* **39,** 207.

CREWTHER, W.G. & LENNOX, F.G. (1950) Preparation of crystals containing protease from *Aspergillus oryzae*. *Nature* (*Lond.*), **165,** 680

FOLIN, O. & CIOCALTEU, V. (1927) On tyrosine and tryptophane determinations in proteins. *J. Biol. Chem.* **73,** 627.

KABAT, E.A. & LANDOW, H. (1942) A quantitative study of passive anaphylaxis in the guinea-pig. *J. Immunol.* **44,** 67.

KABAT, A.E. & MAYER, M.M. (1964) In: *Experimental Immunochemistry*, 2nd edn, pp. 556–558. Charles C. Thomas, Springfield, Illinois.

KASHIWAGI, N., BRANTIGAN, C.O., BRETTSCHNEIDER, L., GROTH, C.G. & STARZL, T.E. (1968) Clinical reactions and serologic changes after the administration of heterologous antilymphocytes globulin to human recipients of renal homografts. *Ann. intern. Med.* **68,** 275.

KASS, E.H., SCHERAGO, M. & WEAVER, R.H. (1946) The effect of enzymatic digestion upon the antigenic properties of antitoxic and normal horse plasma. *J. Immunol.* **45,** 87.

ORIOL, R., BINAGHI, R. & BOUSSAC-ARON, Y. (1968) Preparation d'anticorps monospecifiques anti-immunoglobulines. *Ann. Inst. Pasteur*, **114,** 52.

OVARY, Z. (1958) Immediate reactions in the skin of experimental animals provoked by antibody–antigen interaction. *Progr. Allergy*, **5,** 459.

PORTER, R.R. (1959) The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. *Biochem. J.* **73,** 119.

ROWE, D.S. (1962) The separation and antigenic characteristics of some fractions of papain digests of human serum gamma globulins of some other mammalian sera. *Immunology*, **5,** 533.

SCHEIDEGGER, J.J. (1955) Une micro-methode de l'immune-electrophorése. *Int. Arch. Allergy*, **7,** 103.

SCHULTZE, H.E., HAUPT, H., HEIDE, H., HEIMBURGER, M. & SCHWICK, H.G. (1965) Comparative investigations of purified diptheria and tetanus T-components and their fragments. *Immunochemistry*, **2,** 273.

TERASAKI, P.I. & MCCLELLAND, J.D. (1964) Microdroplet assay of human serum cytotoxins. *Nature* (*Lond.*), **204,** 998.

TORISU, M., SONOZAKI, H., INAI, S. & ARATA, M. (1970) Deficiency of the fourth component of complement in man. *J. Immunol.* **104,** 728.

VAN ROOD, J.J. & VAN LEEUWEN, A. (1963) Leukocyte grouping. A method and its application. *J. clin. Invest.* **42,** 1382.

WEIL, A.J., PARFENTJEV, I.A. & BOWMAN, K.L. (1938) Antigenic qualities of antitoxins. *J. Immunol.* **35,** 399.